BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

IMAGES ARE BEST AVAILABLE COPY.

As rescanning documents *will not* correct images, please do not report the images to the Image Problem Mailbox.

Bone-Marrow Derived Cells for Enhancing Collateral Development: Mechanisms, Animal Data, and Initial Clinical Experiences

Tim Kinnaird, MD, Eugenio Stabile, MD,
Mary Susan Burnett, PhD, Stephen E. Epstein, MD

Cardiovascular Research Institute, MedStar Research Institute,
Washington Hospital Center, Washington, DC, USA

Corresponding Author:
Stephen E. Epstein MD,
Suite 4B-1,
Cardiovascular Research Institute,
Washington Hospital Center,
110 Irving Street NW
Washington
DC 20010

Tel 202877-5977
Fax 202 877-2715
Email: stephen.epstein@medstar.net

Initial animal studies of single angiogenic agents--such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF)--generated enthusiasm for the concept that these agents might enhance collateral development and thereby provide alternative therapies for patients with vascular disease not amenable to traditional revascularization. The enthusiasm, apparently justified by the subsequent results of small non-randomized phase-I clinical trials, was then tempered by the subsequent disappointing results of randomized clinical trials.[1-3] In light of these disappointing results, investigators have pursued alternative strategies in an attempt to improve tissue perfusion. One such strategy is the utilization of bone marrow-derived cell therapy. This review discusses mechanistic pathways mediating the effects of such cell therapy, summarizes the animal and early clinical experience, and speculates on the potential of genetic manipulation of bone marrow-derived cells in an attempt to further enhance their potency.

Angiogenesis vs. arteriogenesis

Two distinct post-embryogenesis mechanisms are responsible for new blood vessel formation in adult life: 1) angiogenesis, where local capillaries proliferate and thereby increase local distribution of blood flow,[4-5] and 2) arteriogenesis, where pre-existing high resistance collaterals enlarge, thereby decreasing flow resistance and increasing total flow to an ischemic region.[6]

Angiogenesis occurs under multiple conditions, including local ischemia. One mechanism by which ischemia leads to angiogenesis is though HIF-1 signaling.[7] Cellular hypoxia stabilizes the HIF-1α transcription factor leading to the expression of many genes involved in the response of the cell to hypoxia including vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), VEGF receptors, nitric oxide, and insulin-like growth factors.[8] In response to changes in local concentrations of these and other cytokines, endothelial cells loosen their intra-cellular connections, sprout, migrate and form new thin-walled vessels.[9,-11] Accompanying these cellular events, local increases in vessel permeability and basement membrane composition occur mediated by local changes in proteases such as MMP-9 and plasminogen activator. The extra-cellular matrix also plays an important role in modulating vessel formation by changing its composition (expressing integrins such as $\alpha_v\beta_3$, which are pro-angiogenic) and structure to induce endothelial cell migration. Thus, sprouting of pre-existing capillaries forms a new immature capillary network. To complete maturation, these newly formed vessels must associate with pericytes, thus ensuring endothelial cell survival, blood flow and vascular permeability. Cytokines playing an essential role in neovasculature maturation include Ang-1, platelet-derived growth factor (PDGF), TGF-β, and PlGF.[12-14]

An increase in the number of capillaries without a concomitant increase in the number and/or caliber of conducting vessels is insufficient to augment tissue perfusion. It is the second mechanism, *arteriogenesis*, which involves remodeling of pre-existing small arterioles into larger vessels, that is the essential component of a developing functional collateral network.[15-16] Under normal circumstances, flow through pre-existing collaterals (lying in parallel to the native artery) is low due to their small caliber and resulting high resistance. However, following parent vessel occlusion, a large pressure drop develops across the pre-existing collateral, resulting in an acute increase in flow and a concomitant increase in shear stress exerted on the vessel wall.[17] This triggers endothelial cell expression of VCAM and ICAM, and the production of several cytokines including monocyte chemoattractant protein-1 (MCP-1), granulocyte-macrophage colony-stimulating factor-10, and tumor necrosis factor-α.[18] In addition, gene array studies in tissue derived from an area of remodeling collaterals demonstrate that the expression of stem

cell-derived factor-1 (SDF-1) is significantly up-regulated.[19] These cytokines recruit circulating monocytes to the activated endothelium, where they adhere, invade the interstitial space and mature to macrophages.[20-21] The macrophages, in addition to other cellular elements, produce abundant cytokines such as VEGF, nitric oxide, more MCP-1, FGF-1 and FGF-2.The new milieu leads to endothelial and smooth muscle cell proliferation, migration, vessel enlargement and maturation, and synthesis of extra-cellular matrix. Ultimately, the small pre-existing arterioles remodel into large functional conducting collaterals.

Mechanistic Considerations

Although an increase in tissue perfusion can only be achieved through efficient arteriogenesis, much of the mechanistic data pertaining to bone marrow-derived cell therapy pertains to angiogenesis. Although there may be considerable mechanistic overlap in the two processes, at this time insufficient information is available to definitively distinguish differences in the molecular processes leading to arteriogenesis and those leading to angiogenesis. Therefore, in the following text we have considered data from studies addressing vascular remodeling and stem cell therapy in general.

Bone Marrow Cells Incorporate Into the Vessel Wall. Many of the cells required to form neovasculature or remodel existing collaterals derive from local proliferation of endothelial and smooth muscle cells.[22-24] However, several studies suggest an additional contribution derived from bone-marrow progenitors, which are mobilized in the setting of limb or myocardial ischemia, migrate to ischemic tissue, and actively incorporate into new vessels.[25-29] It is proposed that tissue ischemia induces local increases in chemokines such as VEGF, thus promoting migration of VEGFR1 and VEGFR2-expressing progenitor cells to the ischemic territory.[30] Thus, one rationale and proposed advantage of cell therapy vs. single agent therapy is that cell therapy directly increases the number of stem/progenitor cells in ischemic tissue. The increase in local stem/progenitor cells could be achieved either by systemic delivery (by systemic injection of the cells or inducing cells to exit the marrow microenvironment by chemokine therapy), or by direct injection of cells into or around the ischemic zone. Regardless of the pathway taken, the number of potential candidate cells to incorporate into the vessel wall would be substantially increased.

Several cell types within the marrow cavity appear to retain the ability to differentiate into one or more of the cellular components of the vascular bed and thus in theory might incorporate directly into the wall of newly formed or remodeled vessels. Endothelial progenitor cells are one such cell type present in the bone marrow (EPCs) and peripheral circulation (CEPs); the role of these cells in angiogenesis is the subject of a separate review.

One bone-marrow cell considered as a potential candidate for transdifferentiation into vascular wall cells is the hematopoietic stem cell (HSC), which displays multi-organ and multi-lineage engraftment in animal marrow irradiation studies.[31] An enriched $CD34^{-/low}$, c-kit^{+}, Sca-1^{+} sub-population of the HSC, termed the side-population (or SP) cell also appears to exhibit transdifferentiation capabilities with incorporation into infarcted myocardium as new cardiomyocytes and endothelial cells.[32-33] The rates of SP incorporation in these studies appears to be relatively low, with only 3% of capillaries in the peri-infarct territory displaying donor cell phenotype. Another HSC derivative - the Lin^{-} *c-kit*^{POS} cell - may also possess multi-lineage

capabilities. In murine acute infarction models, Lin^{-} *c-kit*^{POS} cells directly injected into infarcted myocardium incorporated as cardiomyocytes, and also as endothelial and smooth muscle cells.[34-35] These studies demonstrated higher rates of cell incorporation, with up to 40% of new endothelial cells and smooth muscle cells in the peri-infarct region being derived from donor cells. It appears from these studies that the milieu in which cells find themselves is crucial in directing their ultimate differentiation and commitment to a particular lineage.

The marrow stromal cell (MSC) is another bone-marrow cell type with the potential to incorporate into the developing vascular wall. Marrow stromal cells are also termed mesenchymal stem cells, mesenchymal stromal cells, and mesenchymal progenitor cells, and their nomenclature is the subject of on-going discussion. However, cells isolated by diverse protocols as used by different groups appear to produce cells that are phenotypically and functionally indistinguishable.[36] MSCs do not express cell surface markers typical of hematopoeitic progenitors--such as CD31, CD34, CD45, CD 117 or CD133-- but are uniformly positive for CD90, CD105 and CD166.[37] MSCs retain the ability to differentiate into several mesenchymal lineages including osteoblasts, chondroblasts, and adipocytes.[38-39] However, MicroSAGE analysis also confirms that MSCs express RNA's characteristic of smooth muscle and endothelial cells,[40] and in-vitro studies reveal that after several weeks of culture MSCs acquire a phenotype that closely resembles smooth muscle cells.[41,42] Although most cardiovascular studies of MSC differentiation have focused on their ability to form cardiomyocytes,[43] some evidence of MSC incorporation into neovasculature has been observed.[44] One study directly addressing MSC therapy for angiogenesis suggested that locally delivered MSCs were able to incorporate into newly formed vessels and displayed endothelial and smooth muscle cell phenotypes.[45]

The MSC population consists of a heterogeneous population of cell types. MSC progenitors--termed multipotent adult progenitor cells (MAPCs)--co-purify with MSC cultures.[46-47] Until the isolation of MAPCs, MSCs or their derivatives had not been conclusively demonstrated in-vitro to differentiate into endothelial cell lineages. However, when cultured with VEGF, MAPCs--as well as retaining the typical MSC lineages potential--differentiate into $CD34^{+}$, VE-$cadherin^{+}$, $Flk1^{+}$ cells, a phenotype that would be expected for angioblasts. Subsequently these cells could be induced to express endothelial markers and function as endothelial cells in-vitro.[48] The crucial phenotypic difference between MSC and MAPCs is the expression of CD44. This cell marker is expressed when cultured in greater than 2% serum, and appears to signal MSC commitment away from endothelial cell lineages.

Given the large amount of in-vitro data regarding the plasticity of various bone marrow-derived cell populations, it is tempting to conclude that cell therapy exerts it dominant effects through incorporation into vessels as endothelial or smooth muscle cells. However, the relative importance of direct incorporation into new or remodeling collaterals is the subject of on-going debate, with the magnitude of actual incorporation of donated cells into vascular structures varying substantially between studies.

Although some studies report over half of counted capillaries containing transplanted cells, other studies have reported only occasional positive vessels despite impressive improvements in perfusion.[49-51] Whether *any* cells actually incorporate was examined by inducing hind-limb

ischemia in a mouse previously undergoing marrow irradiation followed by marrow reconstitution by cells derived from a green fluorescent protein (GFP) transgenic mouse. Co-localization of GFP signals with endothelial or smooth muscle cell markers in collateral arteries developing in the ischemic hindlimb was not observed. In contrast, what was observed was an accumulation of GFP^+ fibroblasts, pericytes, and leukocytes adjacent to growing collateral arteries. These results suggest that bone-marrow cells do not promote vascular growth by incorporating directly into the vessel wall, but play an important supportive role.[52] The importance of confocal microscopy was emphasized in this study, as false co-localization of GPF and endothelial signals was observed with standard microscopy.

The issue of adult stem cell plasticity itself has also been questioned, and is currently the subject of intense debate. Single transplanted HSCs were able to reconstitute all peripheral blood components following marrow irradiation, but were not observed in non-hematopoietic tissues, suggesting that transdifferentiation of circulating HSCs and/or their progeny is an extremely rare event.[53] Two very recent studies raised further questions regarding stem cell plasticity in-vivo. In one of these studies, cardiomyocyte-restricted expressed reporter transgenes were used to track the fate of hematopoietic stem cells after 145 transplants into normal and injured adult mouse hearts. Although donor HSCs were observed within myocardial scar tissue, the cells had not transdifferentiated into cardiomyocytes.[54] In the second study, donor cells again were found not to transdifferentiate into cardiomyocytes, but persisted as HSCs within the myocardium and continued to differentiate into blood cells.[55] These studies utilized genetically tagged HSCs, thus avoiding what has been suggested as a potential error introduced by antibody labeling (55a Ken Chien Nature editorial)-one mechanism that may explain the conflicting results of these trials with earlier studies.

Even accepting that donor stem cells are found in non-hematopoietic tissues as differentiated cells, the mechanisms underlying this observation have also been called into question. Co-culture of embryonic stem (ES) cells and murine brain cells expressing a transgenic marker, followed by selection of cells expressing the transgenic marker, recovered a population of undifferentiated cells demonstrating an ES cell phenotype. These cells could be differentiated into multiple cell lineages. However, the demonstration that the undifferentiated cells contained a tetraploid complement confirmed that fusion had occurred.[56] In a similar study, bone-marrow mononuclear cells from transgenic mice expressing both the gene for GFP and puromycin resistance were co-cultured with ES cells.[57] After the addition of puromycin, multiple GFP^+ clones were identified with ES cell morphology. Although these cells were capable of multi-lineage differentiation, they also expressed a haploid karyotype, again confirming that fusion had occurred.

To examine the fusion phenomenon in an animal model, the Cre/lox reporter system (in which activation of Lac-Z expression only occurs following cell fusion) has been used in several studies.[58] Injection of $flox^+/LacZ^+$ myoblasts into Cre(+) murine hearts resulted in Lac-Z positive cells. In contrast, no LacZ positive cells were observed when $flox^+/LacZ^+$ myoblasts were injected into Cre(-) murine hearts. A further study harnessing the Cre/lox reporter model demonstrated bone marrow-derived cells fused spontaneously with cardiomyocytes, resulting in the formation of multinucleated cells. No evidence of transdifferentiation without fusion was observed in this study.[59] Thus, these data reveal the first insights into the potential importance of

progenitor cell/recipient cell fusion. Clearly, any future studies of stem cell therapy will have to convincingly rule out cell fusion before any substantive claims as to plasticity can be made.

Bone-Marrow Cells Function as Supportive Cells.
Despite evidence that bone marrow-derived stem or progenitor cells incorporate into vascular structures, as discussed above, several studies suggest that only a small number of vessels contain donated cells. In addition, heterogeneous bone marrow cell populations (i.e. mononuclear cells or unfractionated cells) contain very small numbers of stem cells (<0.01% of total cells); but despite this, injection of these cells significantly enhances collateral development. Thus, other mechanisms are likely to contribute to the benefits seen following bone marrow cell therapy.

An alternative or perhaps complementary hypothesis to the concept of bone marrow cell incorporation is that the donated cells act in a supportive role, optimizing the milieu for host vasculature to respond to tissue ischemia. Many bone marrow sub-populations are a source of growth factors previously demonstrated to be central in the initiation and coordination of angiogenesis. An early study of bone marrow cell therapy demonstrated that mononuclear marrow cells secrete angiogenic factors such as VEGF and MCP-1 in culture.[60] Other groups confirmed these findings, and observed that following direct myocardial delivery of bone marrow mononuclear cells, increases in cardiac mRNA expression of VEGF, FGF, Ang-1, interleukin-1β and tumor-necrosis factor-α were observed, suggesting localized in-vivo secretion of angiogenic growth factors by the injected cells.[61]

Several stem/progenitor cells are also a source of cytokines, and amongst this group, MSCs are of particular relevance. These cells play a vital supportive role within the marrow micro-environment; their effects are mediated partly through cell-to-cell contact, but also through paracrine signaling. In gene array studies, MSCs express mRNAs for a wide spectrum of angiogenic/arteriogenic cytokines including VEGF, FGF, monocyte chemoattractant protein 1 (MCP-1), placental growth factor, interleukins 1 and 6, insulin-like growth factor, SDF-1, MMP-9 and plasminogen activator.[62] In addition to these cytokines other studies demonstrate that hepatocyte growth factor (HGF) and several insulin growth factors are also released by MSCs.[63-66] Media collected from MSC cultures promoted in-vitro proliferation and migration of endothelial cells and smooth muscle cells, and enhanced collateral flow recovery and remodeling when directly injected into a mouse ischemic hindlimb.[62]

Several other cell populations are potential sources of chemokines. Hematopoietic stem cells express mRNA for VEGF and Ang-1,[32] while EPCs, at least in-vitro, secrete VEGF, HGF, and the potent progenitor cell mitogens granulocyte colony-stimulating factor and granulocyte-macrophage colony-stimulating factor.[67] Interestingly, although another progenitor cell--the angioblast--has not been directly shown to exert paracrine effects, injection of human-derived angioblasts into infarcted rat myocardium stimulated host endothelial cells to proliferate, suggesting that the angioblast may also be a source of pro-angiogenic factors.[68]

Other more mature cellular components of the marrow population also demonstrate potential to exert effects through paracrine mechanisms.[69-70] Several groups have observed that T-lymphocytes release VEGF and play a central role in coordinating the cellular response to

arterial occlusion, in part by recruiting monocytes/macrophages to the site of active collateral artery formation.[71] T-lymphocytes also stimulate endothelial cells to produce VEGF through CD40 signaling, thus providing another pathway via which these cells modulate collateral artery growth.[72] Monocytes, as discussed above, are important mediators of the inflammatory response during arteriogenesis, and are a source of a wide array of chemokines including VEGF, nitric oxide, MCP-1, FGF-1 and FGF-2.[73]

As discussed above, angiogenesis and arteriogenesis are complex processes involving many cellular components and multiple cytokines. These cytokines act not only in a coordinated time and concentration-dependent manner, but one cytokine may potentiate (or inhibit) the effect of another. For example, synergistic relationships between VEGF and bFGF, placental growth factor and VEGF, PDGF and FGF-2, and angiopoietin-1 and VEGF have all been reported.[74-78] In addition, any change in the relative concentrations of one or more of the important cytokines can have catastrophic consequences.[79] FGF-1 when delivered chronically to ischemic myocardium of dogs, led to hemangioma formation.[80] Similarly, injection of VEGF-overexpressing myoblasts caused myocardial hemangiomas in close proximity to the injected cells.[81] It was also demonstrated that micro-environmental concentrations of VEGF were crucial in determining the neovascular response. Below a certain threshold, increased VEGF levels increased the number of morphologically normal capillaries. However, once this threshold was exceeded, increased VEGF resulted in the formation of vascular tumors in every animal treated.[82] In contrast, even a small reduction in VEGF levels may have a profound effect on vascular development.[83]

Thus, it is clear from these data that significant barriers exist in optimizing local cytokine concentrations therapeutically in an attempt to enhance innate responses to tissue ischemia. The theoretical advantage of bone marrow cell therapy over single agent therapy is that as well as delivering vascular cell precursors, such therapy delivers cells that can supply many of the necessary cytokines required to support angiogenesis and arteriogenesis. Although it is tempting to speculate that cell therapy also delivers these cytokines at physiological concentrations in a time-appropriate manner, the large number of cytokines involved and complexity of the processes precludes proof of this concept.

Conclusions regarding potential mechanisms by which bone marrow cell therapy may enhance collateral development.

Controversy continues regarding the exact mechanisms through which bone marrow cells enhance collateral development. Although initial studies suggested that stem cell plasticity and resultant incorporation into the vascular wall was an important phenomenon, more recent data has raised serious concerns regarding its true significance (or indeed occurrence). Furthermore, several groups have independently demonstrated the potential role of supportive mechanisms in mediating the effect of bone marrow therapy for tissue ischemia. However, it is important to bear in mind that the relative balance of incorporation versus paracrine signaling may well vary depending on which cell type is harnessed and in what setting the cells are delivered (see Figure 1 for a summary of the potential mechanisms mediating bone marrow cell effects). Furthermore, uncertainty exists as to which cell is most efficacious, as there is little published data comparing one cell type versus another. Regardless of the gaps in understanding the mechanistic pathways and optimal cell type, the potential of bone marrow-derived cells to affect local arteriogenic

processes via possible transdifferentiation and coordinated secretion of arteriogenic cytokines, make them attractive for interventions aimed at enhancing collateral tissue perfusion.

Bone Marrow Cell Therapy—Pre-Clinical Studies

Several differing approaches to bone marrow cell therapy have been undertaken, with some groups administering heterogeneous populations of cells and other groups selecting specific cell sub-groups (See Figure 2 for a summary of the cell types delivered).

Many small animal studies demonstrate the therapeutic potential of heterogeneous cell delivery for improving collateral flow. For example, transepicardial injection of cultured marrow mononuclear cells (no cell marker data supplied) in a rat cardiac cryo-injury model although designed to study myocardial regeneration, increased capillary counts in the myocardial scar.[50] In a rat model of myocardial ischemia, freshly isolated mononuclear cells increased the number of CD31-positive vessels compared to saline.[55] Following femoral artery ligation, intra-muscular implantation of marrow mononuclear cells increased the number of visible capillaries measured by microangiography, increased hindlimb blood flow estimated by microsphere injection, and improved exercise tolerance by 50% when compared to controls.[84] Mononuclear cell therapy also appeared to augment small and larger vessel remodeling in rabbit hindlimb ischemia, with an increase in capillary count, and enhanced collateral development as assessed by angiographic score and Laser Doppler perfusion imaging.[85] Of particular interest is the demonstration that ex-vivo exposure of mononuclear cells (isolated by density gradient) to hypoxic stress for 24 hours significantly increased capillary count and microsphere-calculated flow recovery when compared to cells cultured under normoxia.[86]

The capacity of mononuclear cell injection to enhance vascular remodeling has also been demonstrated in large animal models. Transendocardial injection of filtered whole bone marrow aspirate in a pig ameroid model of chronic ischemia improved collateral flow as assessed by microspheres.[60] In a porcine acute infarction model, injection of mononuclear cells (isolated by density gradient) resulted in a three-fold increase in the capillary count. This therapy also augmented larger vessel remodeling with a five-fold increase in the number of angiographically visible collaterals, and a reduction in myocardial contrast echo perfusion defect, ultimately leading to a 48% improvement in ejection fraction compared to controls.[61] In a canine chronic coronary occlusion model, transepicardial injection of mononuclear cells following coronary occlusion led to a 50% increase in the number of microvessels observed and a significant improvement in LV wall systolic thickening.[87]

Other groups have examined the role of more selected sub-populations of bone marrow derived cells. Injection of harvested human $CD34^+$ precursors induced by G-CSF injection (most of this sub-set were also $CD117^+$ and contained a high proportion of HSCs and EPCs) led to a five-fold increase in capillary count in a nude rat myocardial infarction model. Echocardiographic myocardial function improved by a mean of 22% compared to controls, with a reduction in the severity of ventricular remodeling observed. These effects persisted to at least 15-weeks post-injection.[68] As discussed, SP cells isolated by Hoechst dye staining ($CD34^-$/low, c-kit^+, Sca-1^+ phenotype) also contribute to infarct healing, incorporating as endothelial and smooth muscle cells, although the effect of this on end-points such as ventricular function and remodeling are less certain.[32,33] HSC derivatives such as the lin^- *c-kit*^{POS} cells have also been utilized in murine

infarct models. Although these cells (isolated from fresh marrow cells by monoclonal antibodies) were observed to incorporate into vascular structures, myocardial regeneration was the most striking finding of this study.[34] Subsequent studies in mice using granulocyte colony-stimulating factor to mobilize lin$^-$ *c-kit*^{POS} HSCs from the marrow cavity prior to the induction of myocardial infarction resulted in significant increase in capillaries, arterioles (with several layers of smooth muscle cells) and myocytes within the scar. The combined effect of this was a 68% reduction in mortality, a 40% reduction in echocardiographic infarct size and significant improvements in post-mortem myocardial hemodynamics.[35]

MSCs also are effective in augmenting the vascular response to arterial occlusion, increasing capillary counts and hindlimb collateral flow.[45,88] In a mechanistic study of cellular arteriogenic potential, MSC (CD34$^-$, CD45$^-$ CD90$^+$ and CD105$^+$ cells selected by magnetic bead sorting from cultured murine marrow) injection increased limb perfusion when measured by laser Doppler, and increased conductance vessel number and total cross-sectional area.[62,89] MAPCs, as might be expected given their clear multi-lineage potential, also contribute to new vessel formation, although this potential has only been exploited in wound healing and tumor angiogenesis models.[48]

In the animal studies conducted thus far, abnormal tissue development subsequent to injection (e.g. bone, cartilage, or teratoma formation), or increased cardiac fibrosis, have not been demonstrated. Accordingly, the safety and suggestive potential efficacy results of these pre-clinical studies served as the basis for several on-going clinical trials.

Bone Marrow Cell Therapy—Preliminary clinical experience

In the first human study, mononuclear cells were injected into ungraftable myocardial territories as an adjunct to coronary artery bypass grafting.[90] Postoperative evaluation revealed no adverse effects and suggested improvement in perfusion in injected territories in 3 of the 5 patients. In a similar pilot study, AC133$^+$ cells were isolated from mononuclear cells and injected intra-myocardially during CABG. [91] During follow-up, LV function improved in 4/6 patients and perfusion in 5/6. Clearly, as cell therapy was delivered as an adjunct to CABG, and in the absence of control groups, no conclusions can be drawn from these studies as to the effectiveness of the cells themselves. Sole therapy using transendocardial injection of whole filtered marrow aspirate, has been employed as part of several pilot and phase I safety and feasibility studies.[92-94] These small non-randomized studies suggested improvements in angina scores and a reduction stress-induced ischemia.

Intracoronary injection of bone marrow cells is an alternative strategy to deliver cells directly to the myocardium. Autologous mononuclear cells were injected into the target vessel approximately 8 days following primary angioplasty for acute infarction (n=10). Although designed to study the role of cells in myogenesis, some improvements in myocardial perfusion were observed, suggesting a contribution of these cells to collateral vessel formation.[95] In a second similar study, investigators assessed the safety and feasibility of intracoronary injection of bone marrow-derived mononuclear cells and peripheral blood-derived mononuclear cells 4 days after successful angioplasty for AMI (n=20). When patients with restenosis were excluded, flow reserve in the infarct vessels appeared to improve substantially.[96] However, in the absence of randomized control groups in each of these studies, the significance of any conclusions

relating to efficacy are uncertain. Finally, in the only randomized human trial of bone marrow cell therapy performed to date, mononuclear cells were injected into the gastrocnemius muscle of patients with peripheral vascular disease, resulting in significant improvements in the ankle-brachial index of the bone marrow treated leg when compared to the control leg treated with peripheral blood-derived cells.[97]

Although encouraging, the data emerging from these preliminary trials should be cautiously interpreted. It is well recognized that patients enrolled in such trials experience a powerful placebo effect. Additionally, no trial thus far has employed an adequately powered, randomized, double-blinded design, and therefore the results of such studies must be cautiously interpreted.

Bone Marrow Cell Therapy—Future directions

As discussed above there are significant theoretical explanations for the disappointing results of trials employing a single angiogenic agent. Cell therapy, mainly by virtue of the potential of this strategy to supply stem/progenitor cells and multiple angiogenic-related cytokines to the region of developing collaterals, may overcome some of these problems. However, there are data to suggest that cell therapy itself may also have inherent limitations.

One example of such a potential limitation is that transplanted cells may have low survival rates, significantly impacting on their beneficial effects.[98-99] Genetically engineering cells to overexpress pro-survival genes such as Akt may be one approach to overcome this limitation. Transplantation of MSCs overexpressing Akt resulted in a fourfold greater myocardial volume than equal numbers of MSCs transduced with a reporter gene.[100] Another potential limitation to cell therapy is the suggestion that cardiovascular risk factors such as aging may also impair the angiogenic effectiveness of cells derived from the older patient in whom autologous cells are to be injected.[101] Furthermore, even if bone marrow cell therapy is more efficacious than single agent therapy, it may still be insufficient to overcome the inhibitory effects of cardiovascular risk factors on collateral development. One approach to this problem may be to genetically engineer the cells to further enhance their therapeutic potential.

Although little is currently known about genetically engineering bone marrow cells to enhance angiogenesis, extrapolation from studies exploring the potential of genetically engineering other types of cells suggests that this direction might be worth further investigation. For example, transduction of skeletal myoblasts with $VEGF_{165}$ appeared to increase capillary density in the vicinity of the transplanted cells compared to non-transduced cells.[102] Whether this conclusion can be extrapolated to the potential of this approach to enhance collateral vessel development is, of course entirely speculative. Similarly, in a study without a concurrent control group (results were compared to previously performed studies), transduction of endothelial progenitor cells with $VEGF_{164}$ appeared to reduce the number of cells required to improve ischemic limb salvage when injected into the ischemic hindlimbs of athymic nude mice.[103]

In an attempt to develop an optimal angiogenic strategy, using an adenoviral vector, a stable HIF-1α analogue (HIF-1α/VP16, see Ref 104 for a description of this construct) was overexpressed in MSCs resulting in a four-fold increase in MSC VEGF release. Subsequently, in a murine model of hindlimb ischemia local injection of MSCs transduced with HIF-1α/VP16 into the ischemic mouse hindlimb significantly increased collateral perfusion compared to non-

transduced cells (105). Whether such interventions will ultimately translate into clinical benefits, however, remains to be seen.

Conclusions

Administration of autologous bone marrow cells is a novel therapeutic strategy derived from the concept that a more optimal arteriogenic effect can be achieved by delivering multiple angiogenic cytokines and cells to regions of tissue ischemia. Experimental studies suggest that such cells may retain the ability to transdifferentiate into vascular cells. However, in what may be the predominant mechanism, these studies also confirm that many of these cells express multiple angiogenic cytokines that support vascular remodeling. Regardless of precise mechanism, delivery of these cells appears to increase tissue perfusion. Whether cell therapy can overcome several potential limitations, or whether more novel approaches need to be explored, such as genetic modification of these cells, is unknown. The clinical need for arteriogenic interventions, the encouraging early experimental results, and the existence of advanced molecular technologies, will undoubtedly further stimulate investigational efforts to optimize cell-based approaches to tissue ischemia.

REFERENCES

1. Rajagopalan S, Mohler ER, Lederman R, Mendelsohn F, Saucedo J, Goldman C, Blebea J, Macko J, Kessler P, Rasmussen H, Annex B. Regional Angiogenesis With Vascular Endothelial Growth Factor in Peripheral Arterial Disease. Circulation. 2003;108:r87-r92.
2. Simons M, Annex BH, Laham RJ, Kleiman N, Henry T, Dauerman H, Udelson JE, Gervino EV, Pike M, Whitehouse MJ, Moon T, Chronos NA. Pharmacological treatment of coronary artery disease with recombinant fibroblast growth factor-2: double-blind, randomized, controlled clinical trial. Circulation. 2002;105:788-793.
3. Epstein SE, Fuchs S, Zhou YF, Baffour R, Kornowski R. Therapeutic interventions for enhancing collateral development by administration of growth factors: basic principles, early results and potential hazards. Cardiovasc Res. 2001;49:532-542.
4. Risau W. Mechanisms of angiogenesis. Nature. 1997;86:671-674.
5. Carmeliet P. Angiogenesis in health and disease. Nat Med. 2003;9:653-660.
6. Schaper W, Buschmann I. Collateral circulation and diabetes. Circulation. 1999; 99:2224 - 2226.
7. Wang GL, Jiang B-H, Rue EA, Semenza G. Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O_2 tension. Proc Natl Acad Sci U S A. 1995;92:5510–5514.
8. Jiang BH, Rue E, Wang GL, Roe R, Semenza GL. Dimerization, DNA binding, and transactivation properties of hypoxia-inducible factor 1. J Biol Chem. 1996;271:17771–17778.
9. Gale NW, Yancopoulos GD. Growth factors acting via endothelial cell specific receptor tyrosine kinases: VEGF's, angiopoietins, and ephrins in vascular development. Genes Dev. 1999;13:1055-1066.
10. Dvorak HF, Brown LF, Detmar M, et al. Vascular permeability factor/vascular endothelial growth factor, microvascular permeability, and angiogenesis. Am J Pathol. 1995;146:1029-1039.
11. Nabel EG, Yang ZY, Plautz G, et al. Recombinant fibroblast growth factor-1 promotes intimal hyperplasia and angiogenesis arteries in vivo. Nature. 1993;362:844-846.
12. Thurston G, Rudge JS, Ioffe E, Zhou H, Ross L, Croll SD, Glazer N, Holash J, McDonald DM, Yancopoulos GD. Angiopoietin-1 protects the adult vasculature against plasma leakage. Nat Med. 2000;6:460-463.
13. Hirschi K, Rohovsky S, D'Amore P. PDGF, TGF-*b* and Heterotypic Cell–Cell Interactions Mediate Endothelial Cell–induced Recruitment of 10T1/2 Cells and Their Differentiation to a Smooth Muscle Fate. J Cell Biol. 1998;141:805-814
14. Luttun A, Tjawa M, Moons L, Wu Y, Scherrer A, Liao F, Nagy J, Hooper A, Priller J, Klerck B, Compernolle V, Daci E, Bohlen P, Dewerchin M, Herbert J, Fava R, Matthys P, Carmeliet G, Collen D, Dvorak H, Hicklin D, Carmeliet P. Revascularization of ischemic tissues by PlGF treatment, and inhibition of tumor angiogenesis, arthritis and atherosclerosis by anti-Flt1. Nat Med. 2002;8:831-840.
15. Schaper W, Ito WD. Molecular mechanisms of coronary collateral vessel growth. Circ Res. 1996;79:911-919.
16. Gorge G, Schmidt T, Ito BR, Pantely GA, Schaper W. Microvascular and collateral adaptation in swine hearts following progressive coronary artery stenosis. Basic Res Cardiol 1989;84:524-535.

17. Shyy Y-J, Hsieh H-J, Usami S, Chien S. Fluid shear stress induces a biphasic response of human monocyte chemotactic protein 1 expression in vascular endothelium. Proc Natl Acad Sci U S A. 1994;91:4678–4682.
18. Nagel T, Resnick N, Atkinson WJ, Dewey CF Jr, Gimbrone MA Jr. Shear stress selectively upregulates intercellular adhesion molecule-1 expression in cultured human vascular endothelial cells. J Clin Invest.1994;94:885– 891.
19. Lee C, Stabile E, Kinnaird T, Shou M, Devaney,J, Epstein S, Burnett M. Temporal patterns of gene expression after acute hindlimb ischemia in mice - insights into the genomic program for collateral vessel development. J Am Coll Cardiol. 2004;43:474–482.
20. Arras M, Ito WD, Scholz D, Winkler B, Schaper J, Schaper W. Monocyte activation in angiogenesis and collateral growth in the rabbit hindlimb. J Clin Inv. 1998;101:40-50.
21. Heil M, Ziegelhoeffer T, Pipp F, Kostin S, Martin S, Clauss M, Schaper W. Blood monocyte concentration is critical for enhancement of collateral artery growth. Am J Physiol Heart Circ Physiol. 2002;283:H2411-2249.
22. Ito WD,Arras M, Scholz D,Winkler B, Htun P, and Schaper W. Angiogenesis but not collateral growth is associated with ischemia after femoral artery occlusion. Am J Physiol Heart Circ Physiol. 1997;273: H1255–H1265.
23. Takeshita S, Rossow ST, Kearney M, Zheng LP, Bauters C, Bunting S, Ferrara N, Symes JF, and Isner JM. Time course of increased cellular proliferation in collateral arteries after administration of vascular endothelial growth factor in a rabbit model of lower limb vascular insufficiency. Am J Pathol. 1995;147:1649–1660.
24. Virag JI, Murry CE. Myofibroblast and endothelial cell proliferation during murine myocardial infarct repair. Am J Pathol. 2003;163:2433-2440.
25. Asahara T, Murohara T, Sullivan A, Silver M, van der Zee R, Li T, Witzenbichler B, Schatteman G, Isner JM. Isolation of putative progenitor cells for angiogenesis. Science. 1997;275:965-967.
26. Takahashi T, Kalka C, Masuda H, Chen D, Silver M, Kearney M, Magner M, Isner J, Asahara T. Ischemia and cytokine-induced mobilization of bone marrow-derived progenitor cells for neovascularization. Nat Med. 1999;5:434-438.
27. Schatteman GC, Hanlon HD, Jiao C, Dodds SG, Christy BA. Blood-derived angioblasts accelerate blood-flow restoration in diabetic mice. J Clin Invest. 2000;106:571-578.
28. Crosby JR, Kaminski WE, Schatteman G, Martin PJ, Raines EW, Seifert RA, Bowen-Pope DF. Endothelial cells of hematopoietic origin make a significant contribution to adult blood vessel formation. Circ Res. 2000;87:728-730.
29. Shintani S, Murohara T, Ikeda H, Ueno T, Honma T, Katoh A, Sasaki K, Shimada T, Oike Y, Imaizumi T. Mobilization of endothelial progenitor cells in patients with acute myocardial infarction. Circulation. 2001;103:2776-2779.
30. Asahara T, Takahashi T, Masuda H, Kalka C, Chen D, Iwaguro H, Inai Y, Silver M, Isner JM. VEGF contributes to postnatal neovascularization by mobilizing bone marrow-derived endothelial progenitor cells. EMBO J. 1999;18:3964-3972.
31. Krause DS, Theise ND, Collector MI, Henegariu O, Hwang S, Gardner R, Neutzel S, Sharkis SJ. Multi-organ, multi-lineage engraftment by a single bone marrow-derived stem cell. Cell. 2001;105:369-377.

32. Jackson KA, Majka SM, Wang H, Pocius J, Hartley CJ, Majesky MW, Entman ML, Michael LH, Hirschi KK, Goodell MA. Regeneration of ischemic cardiac muscle and vascular endothelium by adult stem cells. J Clin Invest. 2001;107:1395-1402.
33. Goodell MA, Jackson KA, Majka SM, Mi T, Wang H, Pocius J, Hartley CJ, Majesky MW, Entman ML, Michael LH, Hirschi KK. Stem cell plasticity in muscle and bone marrow. Ann N Y Acad Sci. 2001;938:208-218.
34. Orlic D, Kajstura J, Chimenti S, Jakoniuk I, Anderson SM, Li B, Pickel J, McKay R, Nadal-Ginard B, Bodine DM, Leri A, Anversa P. Bone marrow cells regenerate infarcted myocardium. Nature. 2001;410:701-705.
35. Orlic D, Kajstura J, Chimenti S, Limana F, Jakoniuk I, Quaini F, Nadal-Ginard B, Bodine DM, Leri A, Anversa P. Mobilized bone marrow cells repair the infarcted heart, improving function and survival. Proc Natl Acad Sci U S A. 2001;98:10344-10349.
36. Lodie TA, Blickarz CE, Devarakonda TJ, He C, Dash AB, Clarke J, Gleneck K, Shihabuddin L, Tubo R. Systematic analysis of reportedly distinct populations of multipotent bone marrow-derived stem cells reveals a lack of distinction. Tissue Eng. 2002;8:739-751.
37. Majumdar MK, Thiede MA, Mosca JD, Moorman M, Gerson SL. Phenotypic and functional comparison of cultures of marrow-derived mesenchymal stem cells (MSCs) and stromal cells. Cell Physiol. 1998;176:57-66.
38. Pittenger M, Mackay A, Beck S, Jaiswal R, Douglas R, Mosca J, Moorman M, Simonetti D, Craig S, Marshak D. Multilineage potential of adult mesenchymal stem cells. Science. 1999;284:143-147.
39. Prockop DJ. Marrow stromal cells as stem cells for non-hematopoietic tissues. Science. 1997;276:71-74.
40. Tremain N, Korkko J, Ibberson D, Kopen GC, DiGirolamo C, Phinney DG MicroSAGE analysis of 2,353 expressed genes in a single cell-derived colony of undifferentiated human mesenchymal stem cells reveals mRNAs of multiple cell lineages. Stem Cells. 2001;19:408-418.
41. Kashiwakura Y, Katoh Y, Tamayose K, Konishi H, Takaya N, Yuhara S, Yamada M, Sugimoto K, Daida H. Isolation of bone marrow stromal cell-derived smooth muscle cells by a human SM22alpha promoter: in vitro differentiation of putative smooth muscle progenitor cells of bone marrow. Circulation. 2003;107:2078-2081.
42. Galmiche M, Koteliansky, Briere J, Herve P, Charbord P. Stromal cells from human marrow cultures are mesenchymal cells that differentiate following a vascular smooth muscle differentiation pathway. Blood. 1993;66-76.
43. Makino S, Fukuda K, Miyoshi S, Konishi F, Kodama H, Pan J, Sano M, Takahashi T, Hori S, Abe H, Hata J, Umezawa A, Ogawa S. Cardiomyocytes can be generated from marrow stromal cells in vitro. J Clin Invest. 1999;103:697-705.
44. Tomita S, Mickle DA, Weisel RD, Jia ZQ, Tumiati LC, Allidina Y, Liu P, Li RK. Improved heart function with myogenesis and angiogenesis after autologous porcine bone marrow stromal cell transplantation. J Thorac Cardiovasc Surg. 2002;123:1132-1140.
45. Al-Khaldi A, Al-Sabti H, Galipeau J, Lachapelle K. Therapeutic angiogenesis using autologous bone marrow stromal cells: improved blood flow in a chronic limb ischemia model. Ann Thorac Surg. 2003;75:204-209.
46. Jiang Y, Jahagirdar BN, Reinhardt RL, Schwartz RE, Keene CD, Ortiz-Gonzalez XR, Reyes M, Lenvik T, Lund T, Blackstad M, Du J, Aldrich S, Lisberg A, Low WC,

Largaespada DA, Verfaillie CM. Pluripotency of mesenchymal stem cells derived from adult marrow Nature. 2002;418:41-49.
47. Reyes M, Lund T, Lenvik T, Aguiar D, Koodie L, Verfaillie C. Purification and ex vivo expansion of pst-natal human marrow mesodermal progenitor cells. Blood. 2001;98:2615-2625.
48. Reyes M, Dudek A, Jahagirdar B, Koodie L, Marker P, Verfaillie C. Origin of endothelial progenitors in human postnatal bone marrow. J Clin Invest. 2002:109:337-346.
49. Wang JS, Shum-Tim D, Chedrawy E, Chiu R. The coronary delivery of marrow stromal cells or myocardial regeneration: pathophysiological and therapeutic implications. J Thorac Cardiovasc Surg. 2001;122:699-705.
50. Tomita S, Li RK, Weisel RD, Mickle DA, Kim EJ, Sakai T, Jia ZQ. Autologous transplantation of bone marrow cells improves damaged heart function. Circulation.1999;100:II247-II256.
51. Iba O, Matsubara H, Nozawa Y, Fujiyama S, Amano K, Mori Y, Kojima H, Iwasaka T. Angiogenesis by implantation if peripheral blood mononuclear cells and platelets into ischemic limbs. Circulation. 2002;106:2019-2025.
52. Ziegelhoeffer T, Fernandez B, Kostin S, Heil M, Voswinckel R, Helisch A, Schaper W. Bone Marrow-Derived Cells Do Not Incorporate Into the Adult Growing Vasculature. Circ Res. 2004;94:230-238.
53. Wagers AJ, Sherwood RI, Christensen JL, Weissman IL. Little evidence for developmental plasticity of adult hematopoietic stem cells. Science. 2002;297:2256-2259.
54. Murry CE, Soonpaa MH, Reinecke H, Nakajima H, Nakajima HO, Rubart M, Pasumarthi KB, Ismail Virag J, Bartelmez SH, Poppa V, Bradford G, Dowell JD, Williams DA, Field LJ. Haematopoietic stem cells do not transdifferentiate into cardiac myocytes in myocardial infarcts. Nature. 2004 Mar 21 [Epub ahead of print].
55. Balsam LB, Wagers AJ, Christensen JL, Kofidis T, Weissman IL, Robbins RC. Haematopoietic stem cells adopt mature haematopoietic fates in ischaemic myocardium. Nature. 2004 Mar 21 [Epub ahead of print].
56. Terada N, Hamazaki T, Oka M, Hoki M, Mastalerz DM, Nakano Y, Meyer EM, Morel L, Petersen BE, Scott EW. Bone marrow cells adopt the phenotype of other cells by spontaneous cell fusion. Nature. 2002;416:542-545.
57. Ying QL, Nichols J, Evans EP, Smith AG. Changing potency by spontaneous fusion. Nature. 2002;416:545-548.
58. Reinecke H, Minami E, Poppa V, Murry CE. Evidence for Fusion Between Cardiac and Skeletal Muscle Cells. Circ Res. 2004 [Epub ahead of print].
59. Alvarez-Dolado M, Pardal R, Garcia-Verdugo JM, Fike JR, Lee HO, Pfeffer K, Lois C, Morrison SJ, Alvarez-Buylla A. Fusion of bone-marrow-derived cells with Purkinje neurons, cardiomyocytes and hepatocytes. Nature. 2003;425:968-973.
60. Fuchs S, Baffour R, Zhou YF, Shou M, Pierre A, Tio FO, Weissman NJ, Leon MB, Epstein SE, Kornowski R. Transendocardial delivery of autologous bone marrow enhances collateral perfusion and regional function in pigs with chronic experimental myocardial ischemia. J Am Coll Cardiol. 2001;37:1726-1732.
61. Kamihata H, Matsubara H, Nishiue T, Fujiyama S, Tsutsumi Y, Ozono R, Masaki H, Mori Y, Iba O, Tateishi E, Kosaki A, Shintani S, Murohara T, Imaizumi T, Iwasaka T. Implantation of bone marrow mononuclear cells into ischemic myocardium enhances

collateral perfusion and regional function via side supply of angioblasts, angiogenic ligands, and cytokines. Circulation. 2001;104:1046-1052.
62. Kinnaird T, Stabile E, Burnett MS, Lee CW, Barr S, Fuchs S, Epstein SE. Marrow-Derived Stromal Cells Express Genes Encoding a Broad Spectrum of Arteriogenic Cytokines and Promote In-Vitro and In-Vivo Arteriogenesis Through Paracrine Mechanisms. Circulation Research. 2004;94:678-685.
63. Weimar IS, Miranda N, Muller EJ, Hekman A, Kerst JM, de Gast GC, Gerritsen WR. Hepatocyte growth factor is produced by human bone marrow stromal cells and promotes proliferation, adhesion and survival of human hematopoietic progenitor cells. Exp Hematol. 1998;26:885-894.
64. Cheng SL, Zhang SF, Mohan S, Lecanda F, Fausto A, Hunt AH, Canalis E, Avioli LV. Regulation of insulin growth factors I and II and their binding proteins in human bone marrow stromal cells by dexamethasone. J Cell Biochem. 1998;17:449-458.
65. Yoon SY, Tefferi A, Li C. Bone marrow stromal cell distribution of basic fibroblast growth factor in chronic myeloid disorders. Haematologica. 2001;86:52-57.
66. Bikfalvi A, Han ZC. Angiogenic factors are hematopoietic factors and vice versa. Leukemia. 1994;8:523-529.
67. Rehman J, Li J, Orschell C, March K. Peripheral blood endothelial progenitor cells are derived from monocyte/macrophages and secrete angiogenic growth factors. Circulation. 2003;107:1164-1169.
68. Kocher AA, Schuster MD, Szabolcs MJ, Takuma S, Burkhoff D, Wang J, Homma S, Edwards NM, Itescu S. Neovascularization of ischemic myocardium by human bone marrow derived angioblasts prevents cardiomyocyte apoptosis, reduces remodeling, and improves cardiac function. Nat Med. 2001;7:430-436.
69. Blotnick S, Peoples GE, Freeman MR, Eberlein TJ, Klagsbrun M. T lymphocytes synthesize and export heparin binding epidermal growth factor-like growth factor and basic fibroblast growth factor, mitogens for vascular cells and fibroblasts. Proc Natl Acad Sci USA. 1994;91:2890-2894.
70. Mohle R, Green D, Moore MA, Nachman RL, Rafii S. Constitutive production and thrombin induced release of vascular endothelial growth factor by human megakaryocytes and platelets. Proc Natl Acad Sci USA. 1997;94:663-668.
71. Stabile E, Burnett MS, Watkins C, Kinnaird T, Bachis A, la Sala A, Miller JM, Shou M, Epstein SE, Fuchs S. Impaired arteriogenic response to acute hindlimb ischemia in CD4-knockout mice. Circulation. 2003;108:205-210.
72. Melter M, Reinders ME, Sho M, Pal S, Geehan C, Denton MD, Mukhopadhyay D, Briscoe DM. Ligation of CD40 induces the expression of vascular endothelial growth factor by endothelial cells and monocytes and promoted angiogenesis in vivo. Blood. 2000;96:3801-3808.
73. Ito WD, Arras M, Winkler B, Scholz D, Schaper J, Schaper W. Monocyte chemotactic protein-1 increases collateral and peripheral conductance after femoral artery occlusion. Circ Res. 1997;80:829–837.
74. Asahara T, Bauters C, Zheng LP. Synergistic effect of vascular endothelial growth factor and basic fibroblast growth factor on angiogenesis in vivo. Circulation. 1995;92:365-371.
75. Carmeliet P, Moons L, Luttun A, Vincenti V, Compernolle V, De Mol M, Wu Y, Bono F, Devy L, Beck H, Scholz D, Acker T, DiPalma T, Dewerchin M, Noel A, Stalmans I, Barra A, Blacher S, Vandendriessche T, Ponten A, Eriksson U, Plate KH, Foidart JM,

Schaper W, Charnock-Jones DS, Hicklin DJ, Herbert JM, Collen D, Persico MG. Synergism between vascular endothelial growth factor and placental growth factor contributes to angiogenesis and plasma extravasation in pathological conditions. Nat Med. 2001;7:575-583.

76. Cao R, Brakenhielm E, Pawliuk R, Wariaro D, Post MJ, Wahlberg E, Leboulch P, Cao Y. Angiogenic synergism, vascular stability and improvement of hindlimb ischemia by a combination of PDGF-BB and FGF-2. Nat Med. 2003;5:603-613.
77. Chae JK, Kim I, Lim ST, Chung MJ, Kim WH, Kim HG, Ko JK, Koh GY. Coadministration of angiopoietin-1 and vascular endothelial growth factor enhances collateral vascularization. Arterioscler Thromb Vasc Biol. 2000;20:2573-2578.
78. Zhou YF, Stabile E, Walker J, Shou M, Baffour R, Yu Z, Rott D, Yancopoulos G, Rudge J, Epstein S. Effects of gene delivery on collateral development in chronic hypoperfusion: diverse effects of Angiopoietin-1 vs. VEGF. JACC. 2004 [in press].
79. Carmeliet P. VEGF gene therapy: stimulating angiogenesis or angioma-genesis? Nat Med. 2000;6:1102-1103.
80. Banai S, Jaklitsch MT, Casscells W, Shou M, Shrivastav S, Correa R, Epstein SE, Unger EF. Effects of acidic fibroblast growth factor on normal and ischemic myocardium. Circ Res. 1991;69:76-85.
81. Lee R, Springer M, Blano-Bose W, Shaw R, Ursell P, Blau H. VEGF Gene Delivery to Myocardium - Deleterious Effects of Unregulated Expression. Circulation. 2000;102:898-901.
82. Ozawa CR, Banfi A, Glazer NL, Thurston G, Springer ML, Kraft PE, McDonald DM, Blau HM. Microenvironmental VEGF concentration, not total dose, determines a threshold between normal and aberrant angiogenesis. J Clin Invest. 2004;113:516-527.
83. Carmeliet P, Ferreira V, Breier G, Pollefeyt S, Kieckens L, Gertsenstein M, Fahrig M, Vandenhoeck A, Harpal K, Eberhardt C, Declercq C, Pawling J, Moons L, Collen D, Risau W, Nagy A. Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. Nature. 1996;380:435-439.
84. Ikenaga S, Hamano K, Nishida M, Kobayashi T, Li TS, Kobayashi S, Matsuzaki M, Zempo N, Esato K. Autologous bone marrow implantation induced angiogenesis and improved deteriorated exercise capacity in a rat ischemic hindlimb model. J Surg Res. 2001;96:277-283.
85. Shintani S, Murohara T, Ikeda H, Ueno T, Sasaki K, Duan J, Imaizumi T. Augmentation of postnatal neovascularization with autologous bone marrow transplantation. Circulation. 2001;103:897-903.
86. Li TS, Hamano K, Suzuki K, Ito H, Zempo N, Matsuzaki M. Improved angiogenic potency by implantation of ex-vivo hypoxia pre-stimulated bone marrow cells in rats. Am J Physiol. 2002;283:H468-H473.
87. Hamano K, Li TS, Kobayashi T, Hirata K, Yano M, Kohno M, Matsuzaki M. Therapeutic angiogenesis induced by local autologous bone marrow cell implantation. Ann Thor Surg. 2002;73:1210-1215.
88. Al-Khaldi A, Eliopoulos N, Martineau D, Lejeune L, Lachapelle K, Galipeau J Postnatal bone marrow stromal cells elicit a potent VEGF-dependent neoangiogenic response in vivo. Gene Ther. 2003; 10: 621-629.

89. Kinnaird T, Stabile E, Burnett MS, Shou M, Lee CW, Barr S, Fuchs S, Epstein SE. Local Delivery of Marrow-Derived Stromal Cells Augments Collateral Perfusion Through Paracrine Mechanisms. Circulation. 2004;109:1543-1549.
90. Hamano K, Nishida M, Hirata K, Mikamo A, Li TS, Harada M, Miura T, Matsuzaki M, Esato K. Local implantation of autologous bone marrow cells for therapeutic angiogenesis in patients with ischemic heart disease: clinical trial and preliminary results. Jpn Circ J. 2001;65:845-847.
91. Stamm C, Westphal B, Kleine HD, Petzsch M, Kittner C, Klinge H, Schumichen C, Nienaber CA, Freund M, Steinhoff G. Autologous bone-marrow stem-cell transplantation for myocardial regeneration. Lancet. 2003;361:45-46.
92. Fuchs S, Satler LF, Kornowski R, Okubagzi P, Weisz G, Baffour R, Waksman R, Weissman NJ, Cerqueira M, Leon MB, Epstein SE. Catheter-based autologous bone marrow myocardial injection in no-option patients with advanced coronary artery disease - a feasibility study. J Am Coll Cardiol. 2003; 41: 1721-1724
93. Tse HF, Kwong YL, Chan JK, Lo G, Ho CL, Lau CP. Angiogenesis in ischemic myocardium by intramyocardial autologous bone marrow mononuclear cell implantation. Lancet. 2003;361:47-49.
94. Perin EC, Dohmann HF, Borojevic R, Silva SA, Sousa AL, Mesquita CT, Rossi MI, Carvalho AC, Dutra HS, Dohmann HJ, Silva GV, Belem L, Vivacqua R, Rangel FO, Esporcatte R, Geng YJ, Vaughn WK, Assad JA, Mesquita ET, Willerson JT. Transendocardial, autologous bone marrow cell transplantation for severe, chronic ischemic heart failure. Circulation. 2003;107:2294-2302.
95. Strauer BE, Brehm M, Zeus T, Kostering M, Hernandez A, Sorg RV, Kogler G, Wernet P. Repair of infarcted myocardium by autologous intracoronary mononuclear bone marrow cell transplantation in humans. Circulation. 2002;106:1913-1918.
96. Assmus B, Schachinger V, Teupe C, Britten M, Lehmann R, Dobert N, Grunwald F, Aicher A, Urbich C, Martin H, Hoelzer D, Dimmeler S, Zeiher AM. Transplantation of Progenitor Cells and Regeneration Enhancement in Acute Myocardial Infarction (TOPCARE-AMI). Circulation. 2002;106:3009-3017.
97. Tateishi-Yuyama E, Matsubara H, Murohara T, Ikeda U, Shintani S, Masaki H, Amano K, Kishimoto Y, Yoshimoto K, Akashi H, Shimada K, Iwasaka T, Imaizumi T. Therapeutic angiogenesis for patients with limb ischemia by autologous transplantation of bone-marrow cells: a pilot study and a randomized controlled trial. Lancet. 2002;360:427-435.
98. Rando TA, Blau HM. Primary mouse myoblast purification, characterization, and transplantation for cell-mediated gene therapy. J Cell Biol. 1994;125:1275-1287.
99. Gussoni E, Blau H, Kunkel LM. The fate of individual myoblasts after transplantation into muscles of DMD patients. Nat Med. 1997;3:970-977.
100. Mangi AA, Noiseux N, Kong D, He H, Rezvani M, Ingwall JS, Dzau VJ. Mesenchymal stem cells modified with Akt prevent remodeling and restore performance of infarcted hearts. Nat Med. 2003;9:1195-1201.
101. Rauscher FM, Goldschmidt-Clermont PJ, Davis BH, Wang T, Gregg D, Ramaswami P, Pippen AM, Annex BH, Dong C, Taylor DA. Aging, progenitor cell exhaustion, and atherosclerosis. Circulation. 2003;108:457-463.
102. Suzuki K, Murtuza B, Smolenski RT, Sammut IA, Suzuki N, Kaneda Y, Yacoub MH. Cell transplantation for the treatment of acute myocardial infarction using vascular

endothelial growth factor-expressing skeletal myoblasts. Circulation. 2001;104:II207-II212.
103. Iwaguro H, Yamaguchi J, Kalka C, Murasawa S, Masuda H, Hayashi S, Silver M, Li T, Isner JM, Asahara T. Endothelial progenitor cell vascular endothelial growth factor gene transfer for vascular regeneration. Circulation. 2002;105:732-738.
104. Vincent KA, Shyu KG, Luo Y, Magner M, Tio RA, Jiang C, Goldberg MA, Akita GY, Gregory RJ, Isner JM. Angiogenesis is induced in a rabbit model of hindlimb ischemia by naked DNA encoding an HIF-1alpha/VP16 hybrid transcription factor. Circulation. 2000;102:2255-2261.

Legends

Figure 1. Mechanisms by which progenitor cells could enhance collateral development. 1. Supportive: Progenitor cells secrete multiple cytokines, growth factors, and chemokines that could facilitate arteriogenesis by a) influencing the matrix in a way that would be conducive for collateral development, b) inhibit endothelial and smooth muscle cell apoptosis and stimulate their migration and proliferation, and c) recruit pro-arteriogenic inflammatory and progenitor cells; 2. Incorporation: Progenitor cells could directly incorporate into the developing collateral and thereby physically contribute to collateral formation—the biological importance of this mechanism is presently a source of controversy; 3. Fusion: Fusion of progenitor cells with tissue specific cells has been demonstrated, but no data are available suggesting this functionally contributes to collaterogenesis.

Figure 2. Different subpopulations of progenitor cells that have been found to enhance collateral development.